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CALCIUM STORAGE AND DISTRIBUTION IN THE DIGESTIVE GLAND OF *BENSONIA MONTICOLA* (GASTROPODA: PULMONATA): A HISTOPHYSIOLOGICAL STUDY

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The pulmonate digestive gland is so well-mined an area of investigation that the reasons for another study on it seem to rest on shaky ground. The structure of this organ has been well known for over eighty years now; it consists of numerous tubules (acini) surrounding the intestine and separated from one another by interacinar spaces. The acini are constructed of an epithelium of various cell types, and a long, as yet unresolved, controversy has raged over whether there are three kinds or four. Even here, much is to be said in favor of the view of Abolins-Krogis (1970) that all types except calcium cells are different functional manifestations of one basic type. Whatever be the ultimate answer to that, it is undisputed that one of the cell types is the calcium cell. It is also uncontested that in addition to serving the function of digestion, this organ in gastropods serves for calcium storage as well (Manigault, 1939; Abolins-Krogis, 1961). An extensive body of literature (*e.g.*, Abolins-Krogis, 1968; Saleuddin, Miranda, Losada and Wilbur, 1970) attests that this organ also releases calcium and other substances in times of increased demand, such as during shell regeneration. The release of calcium is mediated by the breakdown of calcium spherites within the calcium cells (Abolins-Krogis, 1961). While the enormous contribution of this author to knowledge of these spherites is beyond question, much still remains unknown regarding them. Wilbur (1972, p. 108) states that "the spherites, consisting of both mineral and organic components, merit further attention as calcifying systems." It may be mentioned here that these spherites are also of interest by reason of being intracellular calcifying systems, in contrast to shell and bone.

The present communication attempts to elucidate the mechanism of calcium uptake, storage and distribution by this gland. In sum total the amount of calcium taken up by the mollusc is always greater than that lost in normal physiological processes such as shell formation and excretion, and also its level in the blood is maintained within narrow limits (Greenaway, 1971). This points to some sort of regulatory mechanism. Some exceedingly simple hypotheses have been put forth to account for the observed constancy of blood calcium, such as precipitation during periods of saturation in the blood and solubilization in times of depletion. However, matters may not be as simple as that, and the present work proposes a specialized cellular process that may be capable of finer regulation.

Material and Methods

Specimens of *Bensonia monticola* (Gastropoda: Pulmonata) were collected from the hills surrounding Solan (Himachal Pradesh, India) during the months

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of July-September, 1971–1973. The soil of the locality is not visibly calcareous. The shells were cracked open, and the digestive glands were extirpated and divided into small pieces not exceeding 3 mm in any dimension. These were placed in the required fixative (Zenker, alcoholic Bouin, neutral formalin, and absolute ethanol), dehydrated, infiltrated in paraffin wax and sectioned at 7 μ. The sections affixed to glass microslides were subjected to the following histological procedures: hematoxylin-eosin, iron hematoxylin, and Gomori's trichrome staining (Lillie, 1965). While many histochemical methods were applied, only the following are of relevance here, and were performed as detailed in Pearse (1961) unless stated otherwise: von Kossa method for "calcium"; the same, counter-stained with neutral red; Dahl's alizarin red S method for calcium; purpurin method for calcium (Gurr, 1962); alcian blue method for acid mucopolysaccharides (AMPS); the same, counterstained with neutral red; toluidine blue for AMPS and metachromasia in general; Bensley's permanent toluidine blue method (Gurr, 1962), and the periodic acid-Schiff (PAS) method for other carbohydrates. All tests for calcium were performed on material fixed in acid-free fluids (neutral formalin/absolute ethanol); furthermore, all calcium tests (and certain others, see Observations and Results) were accompanied by decalcification controls effected by immersion of the slides in 6% EDTA for 30 min or 5% HNO_3 for 10 min, followed by a distilled water wash. Photomicrography was done with an Asahi Pentax Spotmatic camera mounted upon a Carl Zeiss Jena research microscope.

Observations and Results

The digestive gland of *Bensonia monticola* is a dark brown structure forming the greater mass of the visceral hump. The intestine excavates a convoluted pathway through this mass, being generally cut across more than once in a given section. The remainder of the space is occupied by the transected tubules (acini), which are constructed of an epithelium one cell in thickness. Most of the cells are distally vacuolated. The acini are separated by interacinar spaces which originate beneath the intestinal epithelium. A section of this gland, if stained for calcium, shows the peripheries of the acini brightly outlined with it (Fig. 1). At low magnification it may seem that these deposits lie in the interacinar spaces, but it is very clear at higher magnifications (Fig. 2) that they lie within the calcium cells. They are in the form of spherites, generally not more than five per cell. Each stains dense black with the von Kossa method and bright, fire-orange with the alizarin and purpurin methods. They are completely eliminated by decalcification of the sections prior to staining. The spherites occupy a basal or nearly basal position in the pyramidal calcium cell, which often terminates in a filiform process on the luminal side of the acinus. There is a large and more or less spherical nucleus with chromatin dots and a single nucleolus. The cytoplasm appears to be progressively less dense towards the luminal pole of the cell. The same illustration (Fig. 2) also shows digestive and excretory cells and the nuclei of the undifferentiated interstitial cells, which give rise to the other cell types. The interacinar space (which originates in the sub-intestinal region) is very clear.

Following the localization of calcium spherites an attempt was made to ascertain whether any acid mucopolysaccharide(s) (AMPS) or other carbohydrate lay in association with them. It was thought reasonable to expect AMPS, but per-

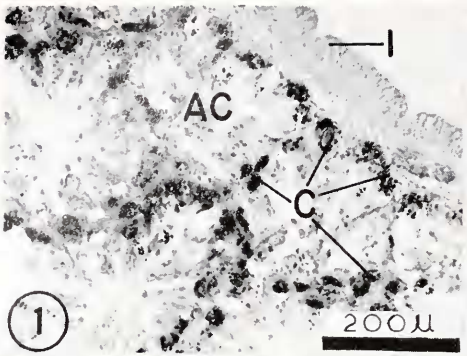
plexingly, staining in alcian blue was either negative or so poor that localization could not be determined. The PAS reaction was also negative. However, one batch of slides was left inadvertently in alcian blue overnight, and it was discovered the next day that a great deal of staining had taken place. Since the normal staining time with alcian blue does not exceed 30 min, it was reasoned that something other than staining was occurring and that this could be decalcification, since alcian blue is made up in 3% acetic acid. Therefore, a fresh set of preparations was run through alcian blue after having been decalcified in EDTA and HNO_3. A remarkable change in the picture occurred, with staining time now being only 15 min (Fig. 3). The whole field was liberally sprinkled with alcianophilic spherules. These spherules were brightly reddish-metachromatic in toluidine blue. They may therefore be interpreted as being granules of AMPS. Closer examination reveals that the spherules are nothing but the organic centers of the calcium spherites left behind after decalcification (Fig. 4), because it is not possible to mistake a calcium cell for any other on account of its distinctive outline and the absence of distal vacuolation. Furthermore, the spherules occupy positions identical to those of the calcium spherites.

Thus the calcium cells are the sites of calcium storage in the digestive gland of *Bensonia*, and the storage is in the form of spherites built around AMPS spherules.

Additionally, there is one more structure of interest, which can be seen whenever sections are stained for calcium. This takes the form of rings which stain dense black in von Kossa's method. Upon close examination it may be confirmed that each ring encloses a blood vessel. This is especially clear when the ring is cut obliquely or longitudinally, and the blood vessel may then sometimes be seen to bifurcate or trifurcate. It may be ascertained that the rings and the branching blood vessels are the same objects in different orientations when one sees that they both contain the same kind of cells—the amoeboid blood corpuscles.

The structure of the rings was enigmatic because they were first discovered in von Kossa-stained sections. This method yields a dense and optically impenetrable black deposit through which no detail may be discerned. It was therefore first thought that the ring represents an amorphous calcium deposit in the form of an ensheathing tube around the blood vessel. The fact that this is not so can be seen when companion sections are stained by alizarin (Fig. 5) and purpurin methods. Here it may be ascertained that each ring is made up of distinct cells whose cytoplasm is choked with calcium granules, and that these cells surround the blood vessels completely. No nucleus was found. In this work these cells have been named the *calcium distributing cells*.

The reason for so naming them is as follows: under high magnifications it can be seen very distinctly that small granules of calcium (as carbonate) lie outside these cells in the basal (interacinar) regions and similar fine granules appear in the lumen of the blood vessels. The cytoplasm of these cells is already full of calcium (Fig. 6). The author has surmised that these cells take up calcium basally and extrude it through the thin and perhaps discontinuous walls of the blood vessels luminally, whence it is borne away by the bloodstream. There can be no confusion in this matter, because the lumen is also seen to contain blood corpuscles (Fig. 6) already mentioned. However, not every individual shows uptake or extrusion or both.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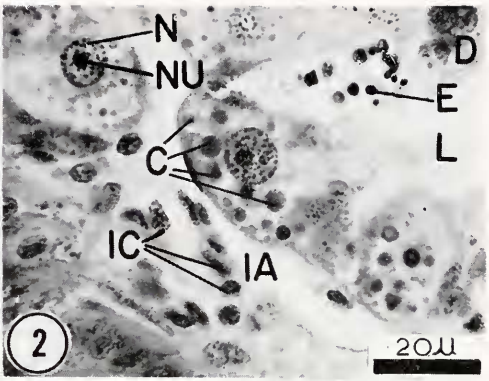
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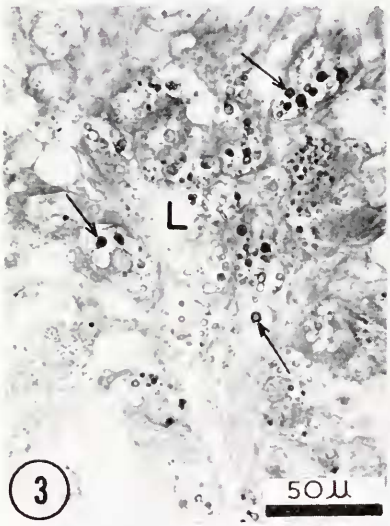
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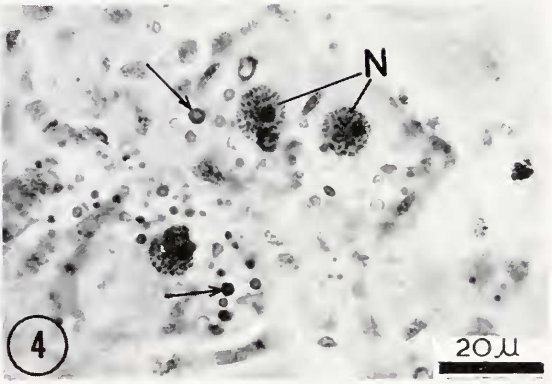
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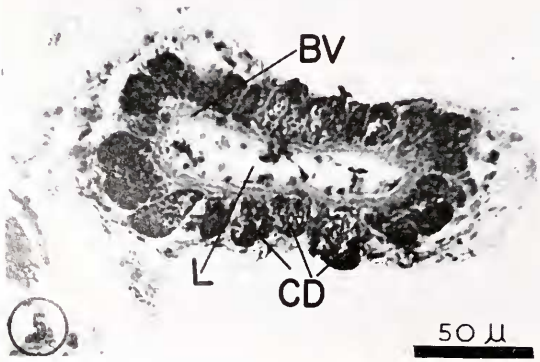
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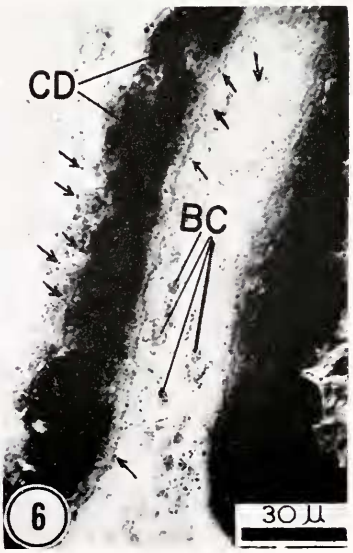
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Finally, although the calcium has been described as granular, it must be mentioned that this description applies to stained sections of fixed material only. There may not be any justification for assuming that transfer occurs in a granular form during life.

Discussion

From the foregoing observations it will be evident that calcium can be localized histochemically in two sites: the calcium cells of the acini and the calcium distributing cells. When food material is present within the lumen of the sectioned intestine, it can also be seen to yield positive reactions for calcium. Though the intestinal epithelium does not stain for calcium, it is easy to surmise that calcium passes through it from the lumen (possibly very rapidly but in histochemically undetectable quantities) into the interacinar spaces. From here it is fairly easy for calcium cells to acquire it through their basal poles.

It is very well known (see Abolins-Krogis, 1961) that these calcium cells do give up their stored calcium in times of need by breakdown of spherites and that the spherites contain AMPS and other organic matter.

Therefore at first sight the calcium distributing cells may seem an organic redundancy, because the functions of storage and distribution are both handled by the calcium cells of the acini. It may also initially seem reasonable that calcium distributing cells are not necessary because once calcium enters the interacinar spaces, it is already in the hemocoel and hence in the bloodstream; any cell serving merely to inject calcium into the bloodstream would appear superfluous.

It may be mentioned here that calcium distributing cells are not being reported for the first time. Greenaway (1971) quotes Carriker (1946) as having found "cells containing calcium deposits lining the outer walls of arteries and capillaries in *Limnaea stagnalis appressa*". This meager information is the only one encountered by the author regarding these cells in published literature, but he is informed (U.

Figure 1. Peripheries of acini (AC) demarcated by abundant calcium deposits (C). The intestine (I) is visible at upper right; neutral formalin/alizarin red S.

Figure 2. Small area from two adjacent acini showing calcium cells and others; the former contain calcium spherites (C) and spherical nuclei (N) with prominent nucleoli (NU). Also seen are a digestive (D) and excretory (E) cell. L is the lumen of acinus at right, while IA marks the well-preserved interacinar space between left and right acini. Nuclei of several interstitial cells (IC) also visible; neutral formalin/von Kossa-neutral red.

Figure 3. Staining with alcian blue following decalcification in EDTA. Numerous AMPS spherules (arrows) stain. The acinus is sectioned longitudinally. L marks its lumen; neutral formalin/alcian blue after decalcification in EDTA.

Figure 4. Three calcium cells after decalcification, stained to show AMPS spherules. Note similarity of their nuclei (N) with those of Figure 2 and that the spherules occupy a position identical to that of calcium spherites in Figure 2; neutral formalin/alcian blue-neutral red after decalcification in EDTA.

Figure 5. Transversely sectioned blood vessel in the digestive gland. Note calcium distributing cells (CD) outside the connective tissue wall of the blood vessel (BV) and blood corpuscles in the lumen (L); neutral formalin/alizarin red S.

Figure 6. Longitudinally-sectioned blood vessel showing intense staining in calcium distributing cells (CD), which are taking up (small arrows at left) particulate calcium and also releasing it into the lumen (arrows in lumen). The nuclei of several blood corpuscles are very clear (BC); neutral formalin/von Kossa-neutral red.

Kanwar, Department of Zoology, Panjab University, personal communication) that rings have also been seen in the digestive gland of another land pulmonate (*Euplecta indica*) from Chandigarh. This would mean that *Bensonia* is only the third pulmonate in which these have been observed. However, no one seems to have speculated upon their function.

The present author believes that the following may be a possible explanation for the calcium pathway in the digestive gland of these pulmonates: calcium is acquired *per os* and enters the interacinar spaces *via* the intestinal epithelium. These are hemocoelic spaces (analogous, roughly, to venous sinusoids), and so the calcium is now in the vascular system. However, the spaces are extensive and dilated in many parts of the body. The movement of hemocoelic fluid within them is sluggish and sufficiently slow to permit the transport of this newly-acquired calcium to the calcium cells in the acini, which take up as much as is required. The interacinar spaces, it is seen, also extend to the rings surrounding the blood vessels, and the surplus calcium is taken up by the calcium distributing cells. In contrast to the situation in the calcium cells of the acini, the calcium here is in a rapidly mobilizable condition, since no spherite breakdown is required and there is no binding organic matrix. The first demand for increased calcium in the blood is met by these calcium distributing cells, which directly inject granular calcium carbonate into the fast-moving blood within the small-bored blood vessels (roughly analogous to arteries and often named as such) which they surround. A greater and more sustained demand (*e.g.*, during shell repair) can be met only slowly by the breakown of calcium spherites within calcium cells of the acini. The calcium from these disintegrated spherites would possibly either slowly percolate through the interacinar spaces and thus fulfill long-term needs or be transferred to the calcium cells for dispersal from there. Besides, the AMPS centers of these spherites would cause firm binding with calcium and permit only slow dissociation. This is indicated by their total inaccessibility to proper staining by alcian blue and their orthochromasia in toluidine blue unless the section is thoroughly decalcified, when their reactive anionic groups are unmasked. The same phenomenon has been seen in reverse order in the pulmonate *Euplecta indica* (Kapur and Sen Gupta, 1970), where the metachromasia of AMPS decreases with progressively greater degrees of calcification, thus indicating more and more binding of Ca^{++} by AMPS. The capability of AMPS to bind metallic ions is well documented (Rao and Goldberg, 1954; Simkiss and Tyler, 1958); also, Horiguchi (1956) and Horiguchi and Miyake (1954) have shown that AMPS extracted from the tissues of *Hyriopsis* and *Pteria* always contain a calcium residue. AMPS are almost universal accompaniments of calcifying systems, being found in such diverse situations as bone, dentine, and enamel in vertebrates, in the egg shells of birds, and in calcified structures of echinoderms, crustaceans, molluscs, sponges, protozoans and even bacteria among the nonvertebrates (for a comprehensive bibliography see Kobayashi, 1971). On the other hand, no such binding occurs in the calcium distributing cells, which present identical pictures in methods for AMPS with or without prior decalcification. In other words, calcium in the calcium distributing cells is highly labile compared to that in the calcium cells of the acini.

A precedent exists in support of the belief that there is a slow- and fast-moving system in the digestive gland. Discussing the turnover of exchangeable tissue

calcium in *Limnaea stagnalis*, Greenaway (1971) stated that exchange of digestive gland calcium appeared to comprise a slow and fast component. He admitted the possibility that the slow component may not represent exchange between blood and digestive gland calcium, but *deposition* of further calcium in the cells of the tissue. This would correspond to the buildup of spherites in the calcium cells in the present instance.

Therefore, it is possible that a mechanism resides in the pulmonate digestive gland for the fine control of blood calcium, and that this has been overlooked for thirty years, since the time of Carriker (1946). Its existence can be proved by the use of autoradiography, but that is beyond the reach of facilities available in this department. This being so, it must be cautioned that the theories regarding calcium uptake and extrusion are based on the appearance of stained sections. There is as yet no experimental evidence in their support; these statements are hypothetical and must be treated as such.

Also, stimuli responsible for the "triggering" of calcium release and the control of its deposition must at this time remain conjectural.

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Summary

The interrelationship of the digestive gland mass and the intestine in a land pulmonate, *Bensonia monticola*, is described. Calcium is found in two principal locations, the calcium cells in the acini and in the calcium distributing cells surrounding the blood vessels. The latter have been barely mentioned in previous literature. The calcium cells in acini contain calcium bound to acid mucopolysaccharides in the form of spherites, whereas the calcium in the calcium distributing cells is unbound, granular and labile. It appears that the latter kind of cell extrudes calcium directly into the blood vessels. The author submits that these two cell types may be responsible respectively for fulfilling slow, long-term needs and rapid, immediate demands, and that together they constitute a fine system of blood calcium regulation that has been overlooked for nearly thirty years now.

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